

In the Specification:

Please make the following changes in the indicated specification paragraphs and sections at the stated pages and line numbers:

Page 2, in the section entitled "REFERENCE TO SEQUENCE LISTING", please make the following changes (latest version in the amendment filed with the joinder papers dated May 7, 2004):

REFERENCE TO SEQUENCE LISTING

A sequence listing appended hereinbelow lists seven sequences for proteins and nucleic acids. The first sequence designated SEQ ID NO: 1 ~~Seq. ID No. 1~~ is for DNA, which codes for the EWS protein of SEQ ID NO: 2 ~~Seq. ID No. 2~~. The sequence designated SEQ ID NO: 2 ~~Seq. ID No. 2~~ is for EWS protein with 656 amino acids. The sequence designated SEQ ID NO: 3 ~~Seq. ID No. 3~~ is an artificial DNA sequence with 20 base pairs. SEQ ID NO: 4 ~~Seq. ID No. 4~~ is an artificial DNA sequence with 21 base pairs. SEQ ID NO: 5 ~~Seq. ID No. 5~~ is an artificial DNA sequence with 27 base pairs. SEQ ID NO: 6 ~~Seq. ID No. 6~~ is an artificial DNA sequence with 33 base pairs and SEQ ID NO: 7 ~~Seq. No. 7~~ is an artificial DNA sequence with 18 base pairs.

A copy of the written sequence listing in computer readable form (CRF) is also provided on an accompanying floppy disk. The content of the sequence listing information recorded in CRF on the floppy disk is identical to the written sequence listings appended hereinbelow and includes no new matter.

Page 12, lines 11 to 16:

EWS protein and EWS-coding ~~EWS-coded~~ nucleic acids are already known in the prior art. Preferably an EWS protein coded by the nucleic acid according to SEQ ID NO: 1 ~~Seq. ID No. 1~~, or its derivative (especially a functional derivative), is ~~are~~ suitable for use in the method according to the present invention. An EWS derivative, which has amino acids 319 to 656 of the sequence described SEQ ID NO: 2 ~~Seq. ID No. 1~~, especially a fragment containing these amino acids, is especially preferred.

Page 12, line 20, to page 13, line 10:

Beyond this the present invention relates to the use of nucleic acids with at least 70 % homology to SEQ ID NO: 1 ~~Seq. ID No. 1~~ or to sequence region 8 to 2032 or sequence region 1000 to 2011 of SEQ ID NO: 1 ~~Seq. ID No. 1~~ for identification and characterization of substances, which influence the activity of nuclear receptors. These types of nucleic acids are cloned in expression cassettes of suitable expression vectors, especially eukaryotic expression vectors.

The term "nucleic acids with at least 70 % homology to SEQ ID NO: 1 ~~Seq. ID No. 1~~" is understood to mean the entire range between 70 % and 100 % homology (also complete correspondence with SEQ ID NO: 1 ~~Seq. ID No. 1~~). The selection of nucleic acids suitable for the respective purpose in the stated homology range is within the ability of those of ordinary skill in the art. The

determination of the nucleic acid homology occurs in a way that is familiar to those of ordinary skill in the art. For this purpose different computer programs, for example BLAST, BLAST-2, ALIGN or MEGALIGN (DNASTAR), are known to those skilled in the art.

Page 14, lines 6 to 11:

A further aspect of the invention accordingly relates to the use of a nucleic acid with at least 70 % homology to SEQ ID NO: 1 ~~Seq. ID No. 1~~, to use of sequence ranges 8 to 2032 or 1000 to 2011 of SEQ ID NO: 1 ~~Seq. ID No. 1~~ or to use of an antibody, which is directed against a protein coded by these nucleic acids, to diagnose illnesses which accompany a dysfunction of nuclear receptor activity, preferably androgen receptor activity.

Page 16, in the "Brief Description of the Drawing" section:

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

The objects, features and advantages of the invention will now be illustrated in more detail with the aid of the following description of the preferred embodiments, with reference to the accompanying figures in which:

Figure 1 is a diagrammatic illustration of the gene for the androgen receptor (AR) and the AR2 fragment, in which the androgen receptor fragment (AR2) is designated as AS: 325 – 919; the activation domain with AF, DNA

binding domains, with DBD; ligand binding domains with LBD; activation domains with AD and binding domains with BD;

Figure 2 is a diagrammatic illustration of the gene for the Ewing sarcoma protein (EWS), in which blue shows the RNA binding domain, dark red, the androgen receptor binding domain AS 219 - 656;

Figure 3 shows the nucleotide amino acid sequence SEQ ID NO: 1 of the EWS protein and the EWS exons of the cDNA coding the EWS protein with the amino acid sequence: SEQ ID NO: 2;

Figure 4 is a graphical illustration showing relative co-activation of the AR signal in SH-SY5Y cells with CMX and EWS respectively; and

Figures 5a and 5b are respective illustrations showing the distribution of EWS transcripts and AR transcripts in various tissues.

Pages 17 to 21, in the "EXAMPLES" section:

EXAMPLES

Example 1:

Oligonucleotides Employed:

Primer for the PCR amplification of library inserts:

Act2c5050Ecc: gattacgctagcttgggtgg (SEQ ID NO: 3 ~~Seq. ID Nr. 3~~)

Act2-4939Xhc: gttgaagtgaacttggcgggg (SEQ ID NO: 4 ~~Seq. ID Nr. 4~~)

Primer for Amplification of EWS-cDNA in full length:

EWS-8-Sal: gggtcgacggacgttgagagaacgagg (SEQ ID NO: 5 ~~SEQ ID Nr. 5~~)

cESW-c2032-Eco: gggaattctgcgggggtctgcacatctagtaggg (SEQ ID NO: 6 ~~SEQ ID-Nr. 6~~)

Sequence Primer:

XII-139a1: gcttgggtgggtcatatgg (SEQ ID NO: 7 ~~SEQ ID-Nr. 7~~)

Vectors Used:

pACT2 (Genbank Access Number U29899) for the library;

pGBT9 derivative for the probes: pGBT9rev and pGBT(+1)rev (Roder, K.H.; Wolf, S.S.; Schweizer, M., 1996, Analytical Biochemistry, 241, pp. 260-62);

pCR2.1 Topo-Vector (Invitrogen Co) for coding of the PCR fragment;

CMX Vector for expression of mammalian cells;

PAIuc for reporter gene assay (contains the MMTV promoter and a Luciferase reporter gene; A. Cato Co.);

pSG5AR (pSG5 with the human genes for the androgen receptor; Gene bank access number AAA51775).

Organisms used:

Yeast strain: Y187 and PJ69-2A

E-Coli Strain: DH5 α

Mammalian Cells: SH-SY5Y (German Collection of Microorganisms and Cell Cultures GmbH (DSMZ): DSM ACC209);
IPC3 (American Type Culture Collection (ATCC): CRL-1435;
and

PC3AR: with pSG5AR stabile transfected PC3 (A. Cato Co.,
Karlsruhe, Germany)

To identify new co-modulators of the androgen receptor a Human cDNA library ("Matchmaker" of Clontech; Nr. HY4028AH) from fetal brain was screened with three different fragments of the androgen receptor (AR) as probe with the help of a yeast-two hybrid system.

For this purpose pSG5AR vector, which contains the cDNA for the human androgen receptor (Genbank AAA51775), was cleaved with the help of Endonuclease PstI, so that three different AR-DNA fragments were produced. The shortest of these fragments (AR4) coded for the N-terminus of the receptor (AS1 – 56), the middle length fragment (AR3) coded for the middle part with the activation domain (AS 57 – 324) and the longest fragment (AR2) coded for the C-terminus (AS 325 – 918) with the DNA and ligand binding domains (DBD and LBD; compare with Fig. 1). AR2 was cloned in the pGBT9(+1)rev vector, since it was previously linearized with the help of endonuclease PstI.

Subsequently the transformation of the pGBT vector, which contains the AR fragment, occurs in the yeast strain PJ69-2A. The positive transformant (Trp+) was incubated with a cDNA library obtained from fetal brain according to the instructions from the manufacturer (Human Multiple Tissue cDNA (MTC), Panel II of Clontech Cat. Nr. K1421-1). 3×10^6 clones were screened in accordance with the instructions from the manufacturer (Clontech). The positive clones were selected and tested for their β -galactosidase activity according to the

instructions of the manufacturer (Clontech). The inserts of the blue colonies originating from the library were increased directly from the yeast cells by means of PCR using the primer Act2c5050Eco and Act 2-4939Xho.

The PCR products were further analyzed by gel electrophoresis for its length after scission and by means of cleavage with MspI. At least one example of each restriction fragment pattern was sequenced using XII-139a1 as sequence primer. The sequences were compared with Incyte of Genbank or Databank.

One of the many identified inserts had a length of 1500 bp and could be identified by sequencing and sequence comparison with Databank NCBJ as coding for the C-terminal part of human EWS (AS 319-656) (see fig. 2 and amino acid sequence in fig. 3).

Fig. 3 shows the nucleotide sequence for the cDNA coding for sequence of human EWS protein together with the derived amino acid sequence. Exons 1 to 17 are shown. The letters printed in bold face characterize the fragment, which is to be found in the yeast two hybrid system and binds to the androgen receptor section AS 325 to AS 919. The sequence regions absent in the splice variant EWS1-b (underlined with a solid line) or EWS1-c (underlined with a dotted line) are underlined in fig. 3.

EWS in its full length was amplified or increased by means of PCR using EWS-8-Sal primer and cEWS-c2032-Eco primer as well as thymus-cDNA or spleen-cDNA of Clontech. The complete coding region of the transcript was isolated from spleen and the variant with exon 15B instead of exon 15 was

isolated from thymus. The amplified cDNA was then cloned with EcoRI and Sal I in the expression cassette of mammalian expression vector CMX.

Figure 4 shows the co-activation of the AR signal in SY-SY5Y cells. 1 µg MMTV-Luciferase and 0.75 µg pSG5AR plasmid were supplied to each reaction chamber of a six-reaction-chamber reaction plate. Each of these six mixtures was transferred to four cavities of a microtiter plate and measured there. The error bars show the standard deviation SD. The measured values were obtained by subtracting the corresponding control values without DHT.

As the bar graph shown in fig. 4 shows, after transient transfection in SH-SY5Y cells EWS is able to induce a strong co-activation of the androgen receptor signal action, especially at low androgen concentrations of 10^{-12} to 10^{-10} mol. For this purpose SH-SY5Y cells in reaction plates with six reaction cavities were transfected with 0.75 µg of a vector, which contained the cDNA for the human androgen receptor (pSG5AR), 1.5 µg of reporter gene construct pAHLuc, which contains the MMTV promoter for the Luciferase gene, and 1 µg of EWS-CMX vector. The transfection occurred using lipofectin of Gibeo BRC according to the instructions of the manufacturer. Twenty-four hours after the transfection the cells were incubated over night with different androgen amounts. The cells were subjected to lysis with a commercial lysis buffer and the luciferase activity was measured in a Lumistar luminometer of BMG Lab Technologies. The EWS-CMX Luciferase activity was compared with the control activity (empty CMX vector). The mixture in each cavity was measured in four cavities of a microtiter plate. The control values of the substance were subtracted without DHT. The

standard deviation was shown with vertical lines indicating the range on the bars in fig. 4.

The tissue distribution of human EWS in normal human tissue is shown in fig. 5a with the aid of autoradiography. Tissues numbered 1 to 16 show the relative amount of human androgen receptor in the following tissues respectively: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testicles, ovaries, small intestine, large intestine and peripheral leucocytes. For this purpose an EWS-cDNA fragment, which coded for amino acids 244 – 656 of EWS, was marked with ^{32}P - α -dATP and the Klenow fragment, according to the MEGAPRIME® Marking system. The marked fragment was purified with a Nick column (Pharmacia) according to the instructions of the manufacturer and was hybridized with Human Blot and Human Northern Blot (MTN) Nr. 7760-1 and Nr. 7759-1 of Clontech. As shown from figure 5a, EWS-RNA is predominantly expressed in testicles. Different tissues contain different amounts of EWS.

Fig. 5b shows the tissue distribution of human androgen receptor in normal human tissues. Tissues numbered 1 to 16 show the relative amount of human androgen receptor in the following tissues respectively: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testicles, ovaries, small intestine, large intestine and peripheral leucocytes. From figs. 5a and 5b one can ascertain the normal expression of both these proteins in tissue.

Figure 5a shows the tissue distribution of EWS transcript (Northern Blot MTN of Clontech). A random priming of the EWS-cDNA fragment of the manufacturer (Amersham), which codes for the amino acids 244 to 656, and marking with ^{32}P - α -dATP and the Klenow fragment took place according to the instructions of the manufacturer. The blots were hybridized with the probe, washed, transferred to a film and developed.